

## Original Investigation

# Association of Cesarean Delivery and Formula Supplementation With the Intestinal Microbiome of 6-Week-Old Infants

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**IMPORTANCE** The intestinal microbiome plays a critical role in infant development, and delivery mode and feeding method (breast milk vs formula) are determinants of its composition. However, the importance of delivery mode beyond the first days of life is unknown, and studies of associations between infant feeding and microbiome composition have been generally limited to comparisons between exclusively breastfed and formula-fed infants, with little consideration given to combination feeding of both breast milk and formula.

**OBJECTIVE** To examine the associations of delivery mode and feeding method with infant intestinal microbiome composition at approximately 6 weeks of life.

**DESIGN, SETTING, AND PARTICIPANTS** Prospective observational study of 102 infants followed up as part of a US pregnancy cohort study.

**EXPOSURES** Delivery mode was abstracted from delivery medical records, and feeding method prior to the time of stool collection was ascertained through detailed questionnaires.

**MAIN OUTCOMES AND MEASURES** Stool microbiome composition was characterized using next-generation sequencing of the 16S rRNA gene.

**RESULTS** There were 102 infants (mean gestational age, 39.7 weeks; range, 37.1-41.9 weeks) included in this study, of whom 70 were delivered vaginally and 32 by cesarean delivery. In the first 6 weeks of life, 70 were exclusively breastfed, 26 received combination feeding, and 6 were exclusively formula fed. We identified independent associations between microbial community composition and both delivery mode ( $P < .001$ ;  $Q < .001$ ) and feeding method ( $P = .01$ ;  $Q < .001$ ). Differences in microbial community composition between vaginally delivered infants and infants delivered by cesarean birth were equivalent to or significantly larger than those between feeding groups ( $P = .003$ ). Bacterial communities associated with combination feeding were more similar to those associated with exclusive formula feeding than exclusive breastfeeding ( $P = .002$ ). We identified 6 individual bacterial genera that were differentially abundant between delivery mode and feeding groups.

**CONCLUSIONS AND RELEVANCE** The infant intestinal microbiome at approximately 6 weeks of age is significantly associated with both delivery mode and feeding method, and the supplementation of breast milk feeding with formula is associated with a microbiome composition that resembles that of infants who are exclusively formula fed. These results may inform feeding choices and shed light on the mechanisms behind the lifelong health consequences of delivery and infant feeding modalities.

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Following birth and the initiation of feeding, the human gastrointestinal tract is colonized by a large diversity of bacterial life. An emerging body of literature in adults has begun to establish clear associations between gut microbiome composition and a wide range of health outcomes.<sup>1-6</sup> In contrast, comparatively little is known about the gut microbiome in infants and children, the exposures that shape it, and its lifelong health effects.<sup>7</sup> Although limited in their size and scope, a number of studies have established associations between intestinal microbiome profiles in infants, delivery mode, and/or breast milk exposure.<sup>8-15</sup> These factors both have long-term health consequences. Cesarean delivery has been associated with an increased risk for obesity, asthma, celiac disease, and type 1 diabetes mellitus,<sup>16-19</sup> whereas breastfeeding has been related to decreased risks for illnesses such as asthma, obesity, infection, metabolic syndrome, and diabetes compared with formula feeding (reviewed in the article by Ip et al<sup>20</sup>). The underlying mechanisms are not well understood, but there is growing evidence linking exposure to microflora that is present during vaginal delivery with the patterns of the microbiome that become established in infants.<sup>21</sup> In addition, following delivery, the feeding of human milk primes and matures the infant gastrointestinal system and is believed to promote a unique microbial colonization profile that has yet to be clearly defined in healthy populations.<sup>22</sup> The acquisition of specific microbes in succession, as the core microbiome of the gut is created, may be permanently affected by exposure to maternal vaginal microflora and/or to breast milk and could represent a key mechanism underlying differences in immune development that influence lifelong disease risk.

The contribution of bacteria through vaginal delivery followed by exclusive breastfeeding promotes specific microbial profiles that facilitate optimal nutrient metabolism and early systemic immune training.<sup>23</sup> The potential short- and long-term effects of perturbations of the gut microbiome of infancy, as influenced by operative delivery or formula feeding, are beginning to be examined. The contribution of the mode of delivery to the infant microbiome has been evaluated.<sup>13,15,24</sup> However, no study has examined the effects of delivery mode and breastfeeding following adjustment for the other, and, to our knowledge, there are few data on the effects of combination feeding (feeding breast milk and formula together). Determining the associations between mode of delivery and breast milk vs formula feeding and microbiome development in infants is critical to informing delivery and feeding decisions or interventions to alter the microbiome for improved health. Our objective was to evaluate the relative associations of delivery and feeding modes with the composition of the intestinal microbiota at approximately 6 weeks of age in 102 infants from a US pregnancy cohort study. The observed differences due to delivery and feeding modes highlight their importance in shaping the early intestinal microbiome and point to possible explanations for some of the risks and benefits associated with infant delivery and feeding practices.

## Methods

### Ethical Approval, Informed Consent, and Privacy

Institutional review board approval was obtained from the Center for the Protection of Human Subjects at Dartmouth Col-

### At a Glance

- We examined the associations between the intestinal microbiome of 6-week-old infants, delivery mode, and feeding method, including supplementation of breast milk feeding with formula.
- We observed significant independent associations between the composition of the infant gut microbiome and both delivery mode and feeding method.
- Differences in microbiome composition between vaginally delivered infants and infants delivered by cesarean birth were greater than feeding method-associated differences.
- Infants fed a diet of both formula and breast milk had a stool microbiome that resembled that of infants fed exclusively formula.

lege, with yearly renewal of approval, and parents provided written informed consent to participate and permit their children to participate.

### The New Hampshire Birth Cohort Study

Pregnant women aged 18 to 45 years were recruited from prenatal clinics, beginning at approximately 24 to 28 weeks' gestation as described previously.<sup>25,26</sup> We performed microbiome characterizations of stool samples collected at approximately 6 weeks of age from full-term infants (>37 weeks' gestational age at delivery, and appropriate growth for gestational age). Six weeks was chosen because it is likely that exclusive breast milk or formula feeding would be well established at this age, and 6 weeks corresponded to routine maternal postpartum visits, a time that allowed for optimal sample collection with minimal participant burden. We evaluated infant diet from birth until the time of stool collection by telephone questionnaires that included questions regarding the duration of breastfeeding and the timing of formula introduction, if any. Infants who were fed breast milk and who had never been given formula prior to the time of stool collection were given the status of exclusive breast milk feeding. Infants who had not been breastfed and who had been fed formula only prior to their stool collection were assigned the status exclusively formula fed. And infants who had received both breast milk and formula prior to their stool collection were identified as having a diet of both breast milk and formula. When possible, we confirmed exclusive breast milk and exclusive formula feeding status using a feeding diary kept by the infants' mothers during the 48-hour period prior to stool collection.

Delivery mode (cesarean vs vaginal delivery) was abstracted from maternal delivery records. Data about infant exposures to medication were derived from questions asked during the telephone questionnaires described here. Mothers were asked whether their infant had received a prescription medication in the first 4 months of life. A free text field was used to record the medication name. If the exact name could not be recalled, as much detail as could be recalled was recorded. Topical medications, including those given for conjunctivitis and antifungals, such as those given for thrush, were not considered. Because antibiotic exposure has been shown to influence the intestinal microbiome,<sup>7</sup> we excluded infants who had received a prescription antibiotic.

### Sample Collection, DNA Extraction, and Sequencing

Study participants provided infant stool samples collected at regularly scheduled maternal postnatal follow-up visits (6 weeks post partum). Stool was aliquoted in sterile tubes and frozen at  $-80^{\circ}\text{C}$  within 24 hours of receipt. Samples were thawed and DNA was extracted using the Zymo DNA extraction kit (Zymo Research). The quantity and purity of the DNA were determined by OD260/280 nanodrop measurement. The reliability and stability of these methods were described by Wu et al.<sup>27</sup> Illumina tag sequencing of the 16S rRNA gene V4-V5 hypervariable region was performed at the Marine Biological Laboratory in Woods Hole, Massachusetts, using established methods.<sup>28,29</sup> Details of sequencing methods, quality control and filtering, and statistical modeling are presented in the eAppendix in the Supplement.

## Results

### Participant Characteristics and Variability and Diversity of the Early Neonatal Microbiome

We evaluated the associations between the composition of the 6-week intestinal microbiome and both delivery mode and

feeding method in 102 full-term, appropriately grown infants enrolled in the New Hampshire Birth Cohort Study. Delivery medical records, telephone surveys, and feeding diaries were used to assess study participant characteristics including delivery mode and feeding method at the time of stool sample collection (Table 1). We found no significant association between delivery mode and feeding method (eTable in the Supplement; Fisher exact test:  $P = .66$ ).

We sequenced the V4-V5 regions of the bacterial 16S rDNA to characterize the microbial communities present in a stool sample from each study participant at 6 weeks of age. Sequencing yielded a total of 14 362 739 bacterial DNA reads (mean, 140 811; range, 27 897-260 579), of which 8 210 402 (mean, 80 494; range, 12 244-178 802) passed quality filters (eAppendix in the Supplement). These were assigned to 241 bacterial genera. More than 90% of reads were represented by 10 genera (Table 2). Stool samples were dominated by *Bacteroides* and *Bifidobacterium* comprising half of sequence reads, with *Streptococcus*, *Clostridium*, *Enterococcus*, *Blautia*, *Veillonella*, *Lactobacillus*, *Staphylococcus*, *Planococcus*, and others representing the remainder (Table 2).

### Associations Between Delivery Mode, Feeding Method, and Microbial Community Composition

Overall stool microbiome community composition was characterized using generalized UniFrac analysis.<sup>30</sup> Controlling for the effects of feeding method, delivery mode was strongly associated with infant gut microbiome composition ( $P < .001$ ;  $Q < .001$ ) (Figure 1A). Likewise, controlling for the effects of delivery mode, the overall association between feeding method and stool microbiome community composition was also statistically significant ( $P = .01$ ;  $Q < .001$ ) (Figure 1B). In pairwise comparisons of the 3 feeding methods, exclusive breastfeeding was associated with a microbiome community distinct from that of infants who were either exclusively formula fed ( $P = .04$ ;  $Q = .05$ ) or fed a combination of breast milk and formula prior to stool collection ( $P = .02$ ;  $Q = .04$ ). There was no statistically significant difference between infants fed a combination of breast milk and formula and those fed exclusively

Table 1. Characteristics of 102 Participants

Variable	Mean (Range)
Gestational age, wk	39.7 (37.1-41.9)
Delivery mode, %	
Vaginal	69
Cesarean	31
Infant sex, %	
Male	54
Female	46
Infant birth weight, g	3530 (2700-4710)
Feeding at 6 wk, %	
Exclusively breastfed	69
Combination feeding	25
Exclusively formula fed	6
Age at formula introduction among combination fed subjects, wk	3.1 (0.1-8.7)

Table 2. Relative Abundance of the 10 Most Abundant Bacterial Genera Identified for All Infants Overall and for Individual Delivery Mode and Feeding Groups

Genus	Group, %					
	Overall (N = 102)	Delivery Type		Feeding Type		
		Vaginal (n = 70)	Cesarean (n = 32)	Exclusively Breastfed (n = 70)	Combination Fed (n = 26)	Exclusively Formula Fed (n = 6)
<i>Bacteroides</i>	26.4	34.6	20.7	27.9	22.1	28.8
<i>Bifidobacterium</i>	22.5	23.3	17.4	25.5	16.8	11.4
<i>Streptococcus</i>	13.8	12.1	14.0	11.7	18.7	16.9
<i>Clostridium</i>	7.9	5.1	8.8	6.8	11.9	2.4
<i>Enterococcus</i>	5.7	4.3	8.7	4.8	6.1	14.6
<i>Blautia</i>	3.6	2.7	5.5	1.8	7.1	9.4
<i>Veillonella</i>	3.4	3.6	4.6	3.5	3.2	2.9
<i>Lactobacillus</i>	3.0	2.5	4.2	3.4	2.8	0
<i>Staphylococcus</i>	2.6	1.6	3.4	3.3	1.2	0.1
<i>Planococcus</i>	2.0	1.4	2.9	1.5	3.3	2.6
Other genera	9.1	8.8	9.8	9.8	6.8	10.9

formula in terms of microbiome composition. There was no significant interaction observed between delivery mode and feeding method ( $P = .49$ ). The lack of an interaction between delivery mode and feeding method remained even after combining exclusively formula fed and combination-fed infants into a single group ( $P = .53$ ).

We calculated within- and between-group average UniFrac distances to assess the group-specific phylogenetic diversity of the microbial communities we observed in our participants. Within-group distances between infants within specific delivery mode and feeding method groups revealed similar average phylogenetic distances among infants who were born vaginally compared with those born by cesarean delivery (Figure 2A). The greatest within-group average pairwise phylogenetic distance was observed among those infants who were fed breast milk supplemented with formula; pairs within this group were on average significantly less similar than pairs within the exclusively breastfed group ( $P = .02$  and  $P = .04$ , respectively), while other comparisons did not reach statistical significance (Figure 2B).

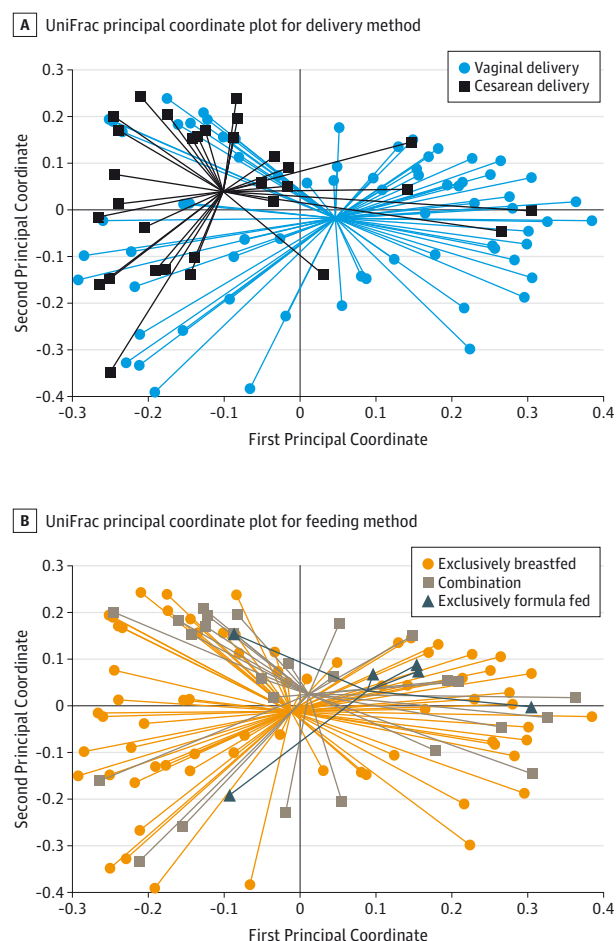
Between-group pairwise UniFrac distances were, on average, as large or larger between vaginally delivered and cesarean-delivered infants as they were between infants from different feeding groups (Figure 2C). For feeding method, average bacterial community phylogenetic distance was greatest between infants who were exclusively breastfed compared with those exclusively formula fed and between infants who were exclusively breastfed compared with those who were fed breast milk supplemented with formula. In contrast, the average distance was smallest between infants who were fed a mix of breast milk and formula and those fed exclusively formula.

We were concerned that some of the participants in the combination-fed group may have been offered the breast in the first few days following delivery but were otherwise effectively exclusively formula fed, which may have driven the difference we observed between exclusively breastfed and combination-fed infants in terms of between-group differences. In fact, of infants who were combination fed in our study, all but 2 were fed breast milk for at least the first 2 weeks of life. To test the robustness of this finding in light of the possible effect of these 2 infants, we repeated the UniFrac analysis after reassigning those 2 infants from the mixed feeding group to the exclusively formula-fed group, and no qualitative differences in the results were observed (data not shown).

### Individual Taxon Abundance by Delivery Mode and Feeding Method

Vaginal delivery (vs cesarean delivery) was associated with increased abundance of *Bacteroides* ( $P < .001$ ;  $Q = .02$ ) and *Pectobacterium* ( $P = .001$ ;  $Q = .02$ ) and with decreased abundance of *Staphylococcus* ( $P = .001$ ;  $Q = .02$ ), *Rothia* ( $P = .006$ ;  $Q = .07$ ), and *Propionibacterium* ( $P = .01$ ;  $Q = .009$ ) in infant stool, after adjustment for feeding method (Figure 3A). Feeding was associated with differential abundance in *Lactococcus* ( $P < .001$ ;  $Q = .002$ ), which was depleted in exclusively breastfed infants compared with those who were exclusively formula fed (Figure 3B). No taxa were significantly differentially abundant between infants who were combination fed vs exclusively formula fed or exclusively breastfed (Figure 3C and D).

Figure 1. Principal Coordinate Plots Comparing Microbial Community Composition Between Delivery Mode and Feeding Method Groups

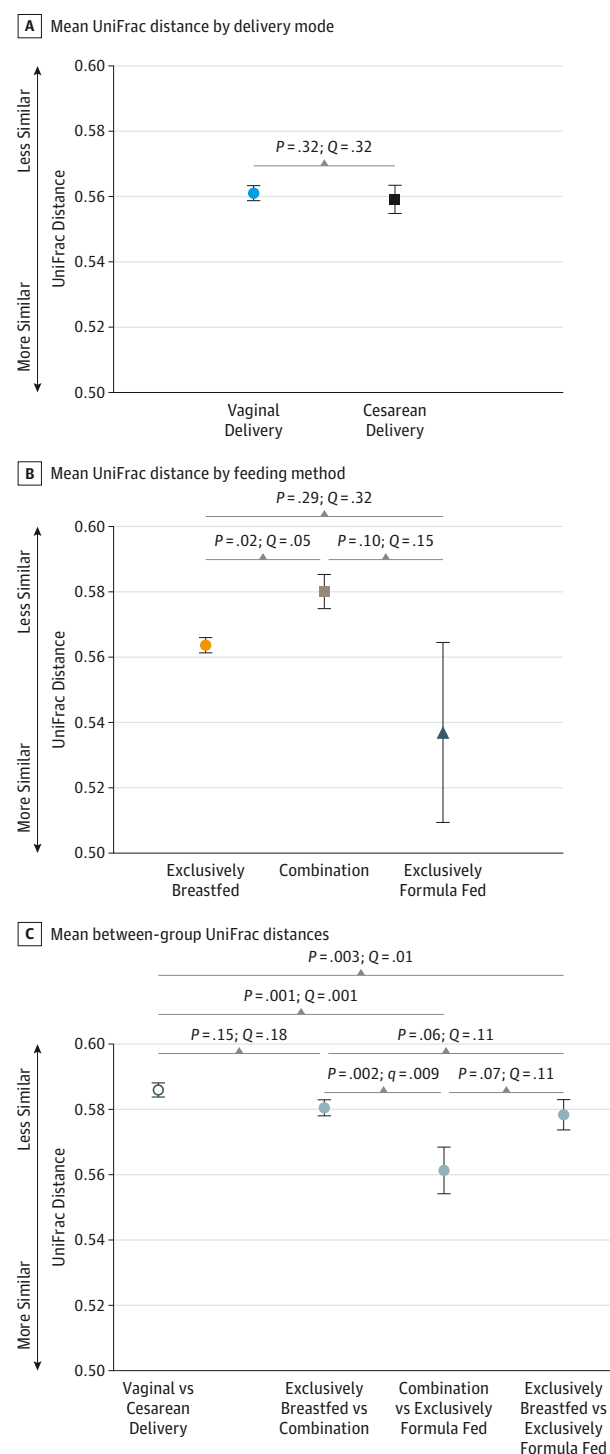


Principal coordinate plots within groups for delivery mode (A) and feeding method (B). UniFrac is a distance metric used for comparing biological communities that incorporates information on the phylogenetic relatedness of community members. Individual participants are represented by points marked according to delivery mode (A) or feeding method (B) and are plotted on the first 2 principal coordinates with permutational multivariate analysis of variance using distance matrices  $P$  values indicated. Lines are drawn from each point to its group centroid. In A,  $P < .001$ ;  $Q < .001$ . In B, the  $P$  and  $Q$  values are as follows: exclusively breastfed vs combination fed ( $P = .02$ ;  $Q = .04$ ), combination fed vs exclusively formula fed ( $P = .52$ ;  $Q = .52$ ), and exclusively breastfed vs exclusively formula fed ( $P = .04$ ;  $Q = .04$ ).  $Q$  values indicate significance of differences after adjusting for multiple comparisons by controlling the false discovery rate for selected comparisons.

## Discussion

We characterized the intestinal microbiome of 102 6-week-old infants and observed independent associations between stool microbial community composition, mode of delivery, and feeding method. In healthy infants, the process of delivery is the initial encounter with microorganisms capable of colonizing the intestinal tract. In a previous study of 24 healthy women, vaginal microbiome composition became less diverse between the second and third trimesters of pregnancy



**Figure 2. Comparison of UniFrac Distances of Microbial Community Composition Between Delivery Mode and Feeding Method Groups**

Mean pairwise UniFrac distances within groups for delivery mode (A), feeding method (B), and between groups (C). UniFrac is a distance metric used for comparing biological communities that incorporates information on the phylogenetic relatedness of community members. Bar height is proportional to mean pairwise UniFrac distance within (A and B) or between (C) groups, with error bars indicating SEM. Throughout, Q values indicate significance of differences after adjusting for multiple comparisons by controlling the false discovery rate for selected comparisons.

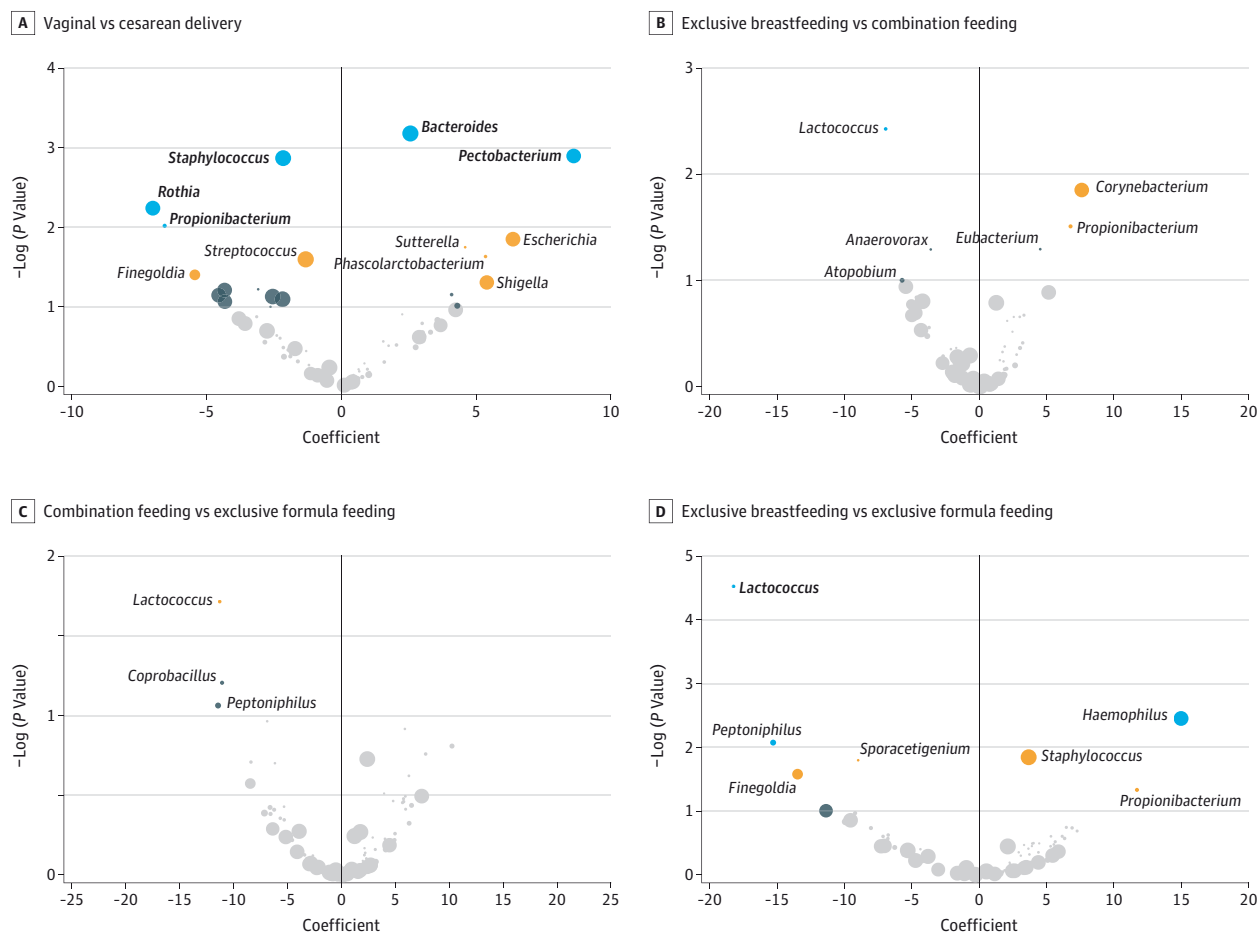
and just before delivery was enriched with *Lactobacillus* species, likely contributing to vertical transmission of these bacteria during vaginal birth.<sup>21</sup> In a study of 10 newborns in Venezuela, within hours of delivery, the intestinal tracts of infants born vaginally were colonized by *Lactobacillus* and *Prevotella*, whereas infants delivered operatively acquired bacteria present on the mother's skin and the hospital environment, such as *Staphylococcus*, *Propionibacterium*, and *Corynebacterium*.<sup>15</sup> Our findings, based on a large group of 6-week-old infants, indicated that *Lactobacillus* also contributes to the microbial environment of the gut but to a lesser extent than *Bifidobacteria*, *Bacteroides*, and *Streptococcus*.

Other studies have observed differences in older infants according to delivery mode. A study of 24 infants aged 3 to 4 months in Canada found that 2 of 26 taxa evaluated were differentially abundant between vaginally and operatively delivered babies, including *Bacteroides*, which was depleted in cesarean-delivered infants relative to those who were vaginally delivered.<sup>9</sup> This result was also observed in a longitudinal study of 24 infants in Sweden, which reported that the depletion of *Bacteroides* in cesarean-delivered infants persisted until 12 months of age.<sup>13</sup> Another longitudinal study of 75 infants in Singapore found that the acquisition of "normal" gut flora was delayed in infants born by cesarean delivery.<sup>31</sup>

To our knowledge, our study was the first to examine the contribution of delivery mode to infant intestinal microbiome composition in association with that of another important predictor of microbiome composition, infant diet. We found that delivery mode was more strongly associated with infant microbiome composition than was diet at 6 weeks. We observed differences in microbial community composition between vaginally delivered and cesarean-delivered infants that were comparable or slightly greater than the largest differences associated with feeding.

We observed an association between feeding method and microbiome composition that remained statistically significant even after adjusting for delivery mode. Although a few previous studies have found associations between infant feeding and intestinal microbiome composition,<sup>9-12,14</sup> to our knowledge, none has examined the relative contribution of combination feeding (breast milk and formula) alongside exclusive formula or breastfeeding to overall microbial community composition. This is an important group to consider because combination feeding is common, for example, in the first few days in the hospital when lactogenesis II is delayed while a mother's breast milk is becoming established, among mothers who have difficulty producing adequate milk and supplement their own milk with infant formula, or among mothers who are unable or choose not to pump breast milk when separated from their babies. We found that the distinction between the microbial communities according to feeding method was largest between infants fed exclusively breast milk and those fed either combination diets or exclusively formula. Infants fed both breast milk and formula had intestinal microbial communities that were similar to those fed exclusively formula and relatively distinct from those fed exclusively breast milk. This finding offers new evidence to support the tenets of the World

Figure 3. Associations Between Individual Genus Abundance and Delivery Mode and Feeding Method



Positive coefficients indicate independent associations with vaginal delivery (A) or breast milk exposure (B-D) after controlling for the other. Point colors correspond to adjusted *P* values of >.10 (light gray), ≤.10, >.05 (dark gray), ≤.05, >.01 (orange), and ≤.01 (blue). Circles are sized according to relative

log-ratio-transformed abundance. The bold labels indicate genera that were significantly differentially abundant after controlling for the false discovery rate at a significance level of *Q* = .10. Note differences in the axis scales.

Health Organization's Baby Friendly Hospital Initiative, which promotes exclusive breast milk feeding beginning at birth in hospitals and birthing centers and the avoidance of formula supplementation unless deemed medically necessary (<http://www.who.int/nutrition/topics/bfhi/en/>). The findings in this study also provide new evidence for pediatricians as they provide guidance to breastfeeding mothers who may be considering incorporating formula into their infant's diet, and they may have implications for decisions around the use of donor human milk in cases when supplementation is needed.

There have been no long-term longitudinal studies of the effects of early feeding method on the microbiome, but early feeding has the potential for lasting effects on microbial community structure,<sup>32</sup> and these effects may be one mechanism for the health benefits of breastfeeding on childhood and lifelong health. Digestion and metabolism of nutrients are likely influenced by the intestinal microbiome,<sup>33</sup> and there is a well-established connection between breastfeeding and lower risk for childhood and adult-onset obesity likely mediated in part by the microbiome in early life (re-

viewed in the study by Thompson<sup>34</sup>). Oligosaccharides in breast milk are thought to promote *Bifidobacterium* growth,<sup>35</sup> and decreased *Bifidobacterium* in infancy has been found to be associated with an increased risk for being overweight at age 10 years.<sup>36</sup> Many formulas are supplemented with prebiotics such as short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides that increase the overall representation of *Bifidobacterium* in the microbiome of formula-fed infants, and similar to breast milk, promote lactate and short-chain fatty acid prevalence in the infant gut (reviewed in the study by Oozeer et al<sup>37</sup>). Although we did not observe a significant association between increased abundance of *Bifidobacterium* and breastfeeding in our study, *Bifidobacterium* was present at greater abundance in exclusively breastfed infants compared with others. Compared with combination-fed infants, this enrichment approached statistical significance before correction for multiple comparisons.

Our conclusions are limited by our study population, which was selected from a single cohort from the United States and

sampled at a single time point; thus, our findings may not be entirely generalizable to populations elsewhere or to different points in infant development. While ours, to our knowledge, is one of the largest studies examining the factors that shape the infant microbiome, our sample size of 102 infants limited our statistical power, which precluded stratified analyses for identifying any interactions between delivery mode and feeding method. In addition, while we were able to categorize feeding practices, the exact proportion of the diet that was made up of either breast milk or formula and the exact timing of formula supplementation (eg, in hospital after delivery vs beginning just prior to 6 weeks) was not considered. It is possible that infants who received formula supplementation only at birth were able to recover a microbiome that resembles that of an exclusively breastfed infant. A previous study highlighted infant nutrition as a major contributor to the early microbiota composition and function, with cessation of breastfeeding contributing the most fundamental shift in the composition of bacteria.<sup>8</sup> A longitudinal study with more participants would allow us to determine the temporal dynamics of the effects of feeding practices and changes therein, as well as the persistence of the effects of both feeding and delivery mode later in infancy. Additionally, exposures, such as postnatal antibiotics, were rare in this cohort and therefore in-

fants with antibiotic exposure were eliminated from analysis. In the future, the evaluation of prenatal, peripartum, and postpartum antibiotic exposure and their role in the trajectory of microbiome development, as well as the interrelationship with delivery mode and dietary exposures, will be important. Thus, our results will need to be replicated in larger multicenter studies and in prospective analyses. While the UniFrac analyses we performed suggest independent associations between microbiome composition and both delivery mode and feeding method, the substantial overlap between the communities defined by both factors suggests that there are other important drivers of microbiome community composition that remain to be identified in future analyses.

## Conclusions

Understanding the patterns of microbial colonization of the intestinal tract of healthy infants is critical for determining the health effects of specific alterable early-life risk factors and exposures. To this end, we have identified measurable differences in microbial communities in the intestinal tracts of infants according to their delivery mode and diet, with possible consequences for both short- and long-term health.

### ARTICLE INFORMATION

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**Author Contributions:** Drs Madan and Hoen had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Drs Madan and Hoen contributed equally.

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